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High Prevalence of Renal Transplant Recipients Infected With More Than One Cytomegalovirus Glycoprotein B Genotype

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A prospective analysis of cytomegalovirus (CMV) glycoprotein B (gB) genotypes was conducted on 34 renal transplant recipients using peripheral blood leukocytes (PBLs) and urine specimens. The CMV gB genotypes were analyzed by polymerase chain reaction (PCR) followed by enzyme digestion. PBLs and urine samples showed almost equal proportions of the 4 known gB genotypes, as well as equal proportions of gB genotype mixtures. The gB genotypes 1, 2 and 3 were equally distributed in the patients. Twenty-four (70.6%) patients had more than one gB genotype during follow-up. There was no association of gB genotypes with the development of symptomatic CMV infection. ***J. Med. Virol. 61:138–142, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: renal transplantation; cytomegalovirus; glycoprotein B; polymerase chain reaction

INTRODUCTION

Human cytomegalovirus (CMV) causes significant morbidity and mortality in renal transplant recipients [Patel and Paya, 1997]. Although 60–100% of renal transplant recipients develop CMV infection, only 20–60% have a symptomatic infection, usually within the first 3 months after transplantation [Mocarski, 1996]. This fact might reflect differences in the degree of immunodeficiency caused by treatment against graft rejection. It has also been suggested that different virus strains may vary in virulence [Brown et al., 1995]. Thus, genetic variation of functionally important CMV genes might influence the severity of disease in renal transplant recipients. One important component of CMV virion is the glycoprotein B (gB). This glycoprotein is not only the major target for neutralizing antibodies [Britt and Alford, 1996], but it is also important for virion penetration into cells, transmission of infection from cell to cell and membrane fusion of infected cells [Navarro et al., 1993]. Analysis of the gene coding gB has shown that clinical isolates adopt one of four sequence configurations, allowing all isolates to be classified in genotypes 1 to 4 [Chou and Dennison, 1991]. Several reports have suggested an association of gB genotypes and pathogenicity in bone marrow transplant (BMT) recipients [Fries et al., 1994; Torok-Storb et al., 1997; Woo et al., 1997]. In AIDS patients contradictory results were found. Shepp et al. [1996] observed an association of gB2 and retinitis that was not confirmed by Peek et al. [1998]. The gB1 genotype were found less frequently, however, in retinitis patients as compared to HIV-positive patients with asymptomatic CMV infection [Bongarts, 1996]. In renal transplant recipients, Woo et al. [1997] and Vogelberg et al. [1996] did not find an association of gB genotypes with severity of disease.

To investigate further the possible role of CMV gB genotypes in the pathogenesis of renal transplant recipients, CMV positive samples obtained at regular intervals from patients living in a region of high prevalence of CMV infection were examined.

MATERIALS AND METHODS

Study Population

Thirty-four consecutive patients who underwent renal transplantation at the Ribeirao Preto General Hospital (RPUH) of Sao Paulo University, between July 1996 and October 1997, all positives for CMV by the polymerase chain reaction (PCR), were enrolled prospectively in this study. Immunosuppressive therapy was started on the day of transplantation with oral administration of cyclosporine (CsA), prednisolone (P), and azathioprine (AZA). Cyclosporine administration was started at a dose of 8 mg/kg/day, and was reduced 1 mg/kg/day weekly until a maintenance dose of 4 mg/kg/day. Prednisolone administration was started at a dose of 1 mg/kg/day for 10 days, reduced to a dose of

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0.75 mg/kg/day for 20 days, 0.5 mg/kg/day for 30 days, and finally a maintenance dose of 0.25 mg/kg/day. Azathioprine was always administered at a dose of 2 mg/kg/day. Whole blood with EDTA as the anticoagulant and urine samples were obtained from patients before transplantation, weekly from patients admitted to hospital after transplantation and at more irregular intervals from outpatients during follow-up. A lung biopsy was also obtained from one patient. The patients were observed for at least 3 months except in cases of graft loss or death.

Clinical and laboratory data were obtained by examination of patients' records at the RPUH. The patients were clustered in two groups, those with symptomatic CMV infection and those with asymptomatic CMV infection, according to Van der Berg et al. [1989] with some modifications. Briefly, the symptomatic CMV infected patients were characterized by the presence of viral DNA detected in peripheral blood leukocytes (PBLs) or urine, and unexplained fever (>37.5°C) for at least 3 days in combination with at least one of the following features: arthralgia, leukopenia ($<3 \times 10^9$/L), thrombocytopenia (150×10^9/L), liver enzyme elevation (ALT >50 U/L), pneumonitis without other causes, or gastrointestinal ulceration. Asymptomatic CMV infected patients had viral DNA detected in PBLs or urine without the signs, symptoms, and laboratory abnormalities stated above. Graft rejection associated with detection of CMV DNA was also analyzed in the patients.

Sample Preparation and DNA Purification

For PBLs separation, 3–5 ml EDTA-anticoagulated whole blood samples were processed immediately after collection. Briefly, 1 ml of 1% dextran was added to each blood sample and the mixture was incubated at 37°C for 30 min. Ten ml of phosphate-buffered saline (PBS) was added to the supernatant fluids and centrifuged at 300 g for 7 min. The PBLs pellets were washed in 10 ml of PBS, centrifuged and suspended in 200 μl of PBS. The lung biopsy sample was prepared for DNA extraction by crushing the tissue in 200 μl of PBS. For DNA extraction 200 μl of PBL, lung biopsy, or untreated urine samples were processed by the Qiamp Blood Kit (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. The DNA samples were resuspended in 200 μl of water.

Amplification of CMV DNA

To reduce the risk of false-positive results, each step of the PCR was undertaken in different places with different pipettes and using tips with filters (Gibco, Grand Island, NY). The PCR was used to amplify a region of high sequence variability in the gB gene of CMV. The reaction mixture having a total volume of 50 μl, contained 5 μl of DNA sample, 50 mM of KCl, 10 mM of Tris-HCl (pH 9), 3 mM of $MgCl_2$, 50 μM each of the dNTPs and 0.3 μM of primers (gB1319 5′TGGAACTGGAACGTTTGGC3′ and gB1604 5′GAAACGCGCGGCAATCGG3′) [Chou and Dennison, 1991]. The mixtures were incubated at 95°C for 3 min in an automated thermal sequencer (Techne, UK), then the temperature was reduced to 80°C and 1 U of Taq DNA polymerase was added (hot start). Amplification of the CMV DNA was undertaken with 15 cycles of 60 sec at 94°C, 120 sec at 65°C, and 120 sec at 72°C, followed by 30 cycles of 60 sec at 94°C, 90 sec at 55°C, and 120 sec at 72°C, including a final extension of 3 min at 72°C. Amplification products (293 to 296 bp, size varied by strain) were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

A high sensitivity semi-nested PCR was carried out in specimens that did not develop a sufficient quantity of material for CMV genotyping. The reaction mixture of the first round of amplification contained 5 μl of DNA sample, 50 mM of KCl, 10 mM of Tris-HCl (pH 9), 3 mM of $MgCl_2$, 50 μM each of the dNTPs and 0.075 μM of the gB1319 primer and the external gB1676 (5′TGACGCTGGTTTGGTTGATG3′) primer designed by Torok-Storb et al. [1997]. Amplification was carried out beginning with the hot start procedure, followed by 25 cycles at 95°C for 60 sec, 55°C for 90 sec, and at 72°C for 120 sec, with a final extension of 3 min at 72°C. Two microliters from the first round of amplification were used as a template in the second round of amplification including the same components except for 0.3 μM of the primers gB1319 and gB1604. Amplification was carried out beginning with the hot start procedure, followed by 30 cycles of 60 sec at 95°C and 60 sec at 65°C, with a final extension of 3 min at 72°C.

Restriction Enzyme Digestion and Fragment Analysis

Five microliters of unpurified PCR products, obtained with primers gB1319–gB1604, were digested with *Hinf* I and *Rsa* I (New England Biolabs, Beverly, MA), in two separate reactions, according to the manufacturer's instructions. Digested fragments were analyzed after electrophoresis on 12% polyacrylamide gels stained with ethidium bromide.

Statistical Analysis

Statistical significance was determined using the Fisher's exact test.

RESULTS

One hundred and ten PBLs, 59 urine, and one lung biopsy samples from the 34 renal transplant recipients with CMV infection was examined. The digest patterns that distinguish gB genotypes 1–4 and the mixture of genotypes are shown in Figure 1. It was found 42 (24.9%) samples having mixture of gB genotypes as shown in Table I. Samples with a unique gB genotype were more frequent as compared to those having a mixture of gB genotypes. The frequencies of mixture of gB genotypes were very similar in PBL and urine specimens.

One hundred thirty-seven CMV genotypings were carried out on the PBLs samples, as well as 72 on the urine samples including those presented as mixture in the same sample. The frequencies of gB 1–4 genotypes

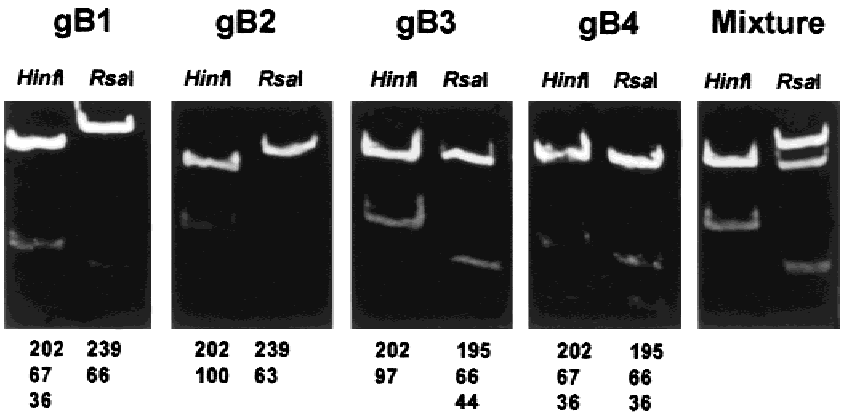

Fig. 1. Restriction digestion with *Rsa*I and *Hinf*I of PCR products obtained with the primer pair gB1319-gB1604. Estimated sizes of digestion fragments are below each lane. An example of mixture of gB2 and 3 genotypes is shown in the last gel.

TABLE I. PBL and Urine Specimens Containing a Single or Mixture of gB Genotypes

	PBL	Urine	Total
Single genotype	80 (72.7%)	47 (80%)	127 (75.1%)
Mixture of genotypes	30 (27.3%)	12 (20%)	42 (24.9%)

observed in PBLs samples were 39.4%, 22.6%, 33.6%, and 4.4%, and in urine samples 41.7%, 20.8%, 36.1%, and 1.4%, respectively. The gB genotypes were distributed equally in PBLs and urine of renal transplant recipients.

The frequencies of gB 1–4 genotypes in the renal transplant recipients were 61.8%, 41.2%, 58.8%, and 8.8%, respectively. Twenty-four (70.6%) patients presented more than one gB genotype during the follow-up (Fig. 2). The distinct gB genotypes were present in either a unique or multiple samples. Ten (29.4%) patients presented only 1 genotype.

Among the 34 patients studied there was one with CMV (gB3) detected just in a pulmonary biopsy and not in PBLs or urine samples in a period of 13 months after renal transplantation. This patient probably had a latent infection and was therefore excluded from the subsequent analysis of CMV genotypes observed in symptomatic and asymptomatic CMV cases.

Seven patients (21.2%) developed CMV symptomatic infection and 2 of these had CMV associated organ disease. One patient with pneumonia and gastrointestinal ulceration was infected with gB 2 and 3, and the other patient with pneumonia was infected with gB 2 and 4. The genotypes observed in symptomatic and asymptomatic patients are shown in Table II. There was no correlation between gB genotypes and clinical presentation of CMV infection.

Considering that most of the renal transplant recipients were infected with more than one gB genotype, the association of combined pairs of gB genotypes with clinical presentation of CMV infection was examined, as shown in Figure 2 and summarized in Table III. There was no correlation of different CMV genotype combinations with symptomatic or asymptomatic cases.

Fourteen patients developed graft rejection, and 8 (57.1%) had viremia before or within the week of graft rejection, that could indicate a participation of CMV in this process. Among these patients it was not found association between gB genotypes and graft rejection ($P > 0.05$). Three patients died, one infected with gB1, one with gB2 and 4, and one with gB2 and 3.

DISCUSSION

Genotyping CMV directly in blood and urine specimens, is a simple and rapid technique that allows the use of stored samples, avoiding the slow and laborious tissue culture processing. It also avoids the possibility of selection of certain virus types during cell culture. To improve the identification of CMV gB genotypes, a high sensitive semi-nested PCR was carried out with samples that did not develop sufficient material for genotyping. Samples of patients containing genotype mixtures that could not be resolved were diluted and PCR was carried out on these dilutions. Thus, all CMV detected in renal transplant recipients were genotyped.

PBLs and urine samples had almost equal proportions of the 4 gB genotypes, as well as equal proportions of gB genotype mixtures. Similar results were also reported by Bongarts et al. [1996] and Meyer-König et al. [1998], indicating that the virus circulating in blood usually appears in urine.

A similar distribution of gB1, 2, and 3 was found in the renal transplant recipients, that is in keeping with the similar distribution observed by our group in another study of newborns and infants from the same region [unpublished data]. These three genotypes seem to be circulating in equal proportion in this region of Brazil.

A high proportion of patients were infected with more than one gB genotype. This data are in contrast to the 96.3% and 90% of patients infected with only one gB genotype reported in previous studies with renal transplant recipients [Woo et al., 1997; Van den Berg et al., 1989]. This difference could be due to the larger number of samples analyzed from each patient in this study, thus facilitating the detection of multiple genotypes during the follow-up. The high proportion of patients infected with more than 1 gB genotype observed in this work, however, could also be explained by the high prevalence of CMV infection in Brazil [Mussi-

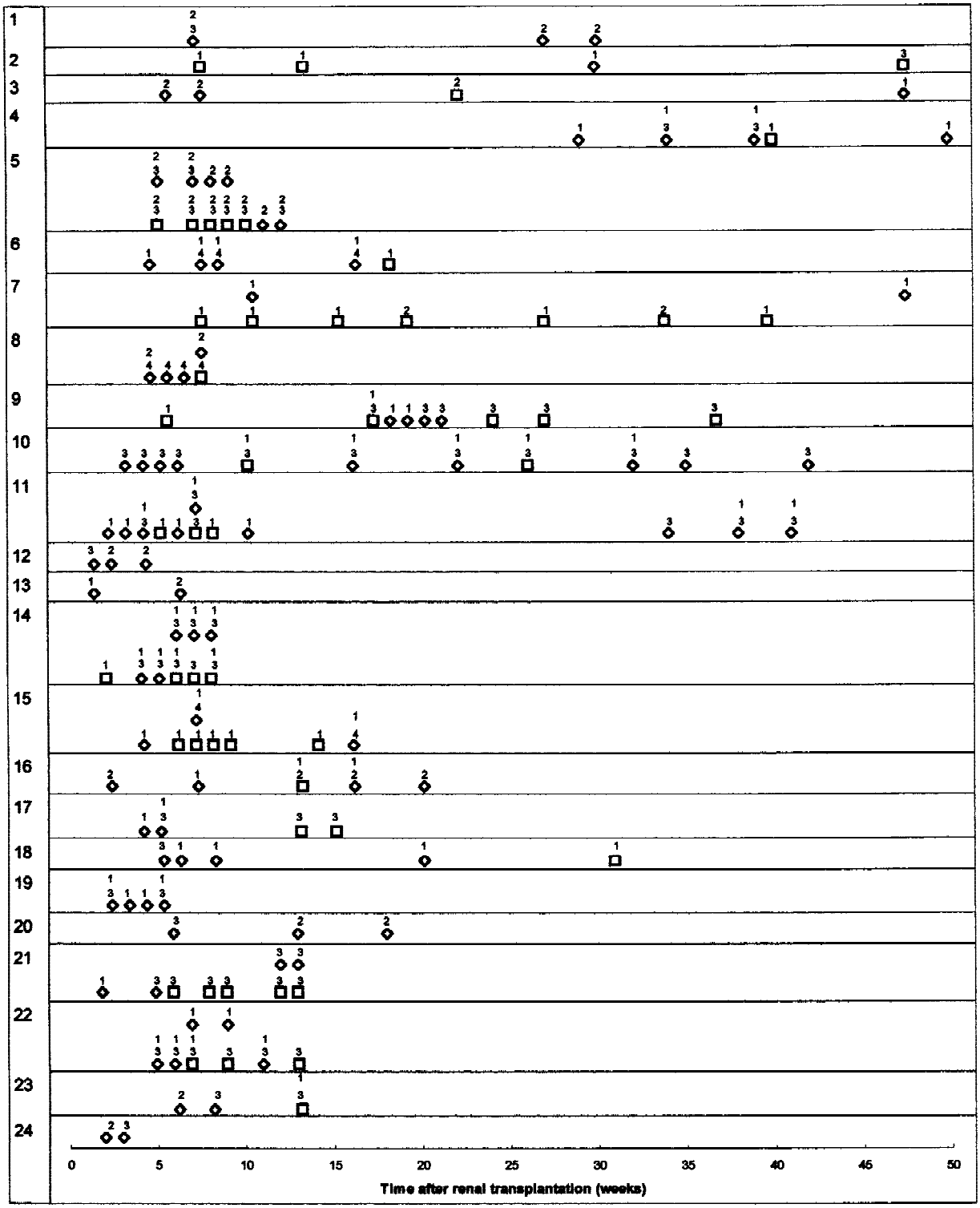

Fig. 2. CMV gB genotypes present in PBLs and urine samples of the 24 renal transplant recipients presenting more than one gB genotype, including those having mixtures of gB genotypes in the same sample. The lozenge represents a PBLs sample and the square an urine sample. The numbers on the geometric figures represent the detected gB genotypes.

Pinhata et al., 1998]. Therefore, the immunocompromised renal transplant recipients could be infected consecutively with several CMV gB genotypes by the use of blood products, during hemodialysis sessions, or by the kidney transplant itself. Unfortunately, it was not possible to have information on the CMV sero status of donors. The prevalence of CMV infection in adults of this Brazilian region, however, is as high as 94–100%

TABLE II. gB Genotypes in Renal Transplant Recipients With Symptomatic and Asymptomatic CVM Infection

	CMV infected patients	
Genotypes	Asymptomatic	Symptomatic
1	19 (70.4%)	2 (28.6%)
2	10 (37.0%)	4 (57.0%)
3	15 (55.6%)	5 (71.4%)
4	2 (7.4%)	1 (4.3%)

TABLE III. gB Genotypes Combination Observed in Renal Transplant Recipients With Symptomatic and Asymptomatic CMV Infection

	CMV infected patients		
Genotypes combination	Symptomatic (n = 5)	Asymptomatic (n = 19)	Total (n = 24)
1–2		4 (21.1%)	4 (21.1%)
1–3	1 (20%)	10 (52.6%)	11 (57.9%)
1–4		2 (10.5%)	2 (10.5%)
2–3	3 (60%)	3 (15.8%)	6 (31.6%)
2–4	1 (20%)		1 (5.2%)

[Mussi-Pinhata et al., 1998]. Frequent infection with multiple gB genotypes was also reported in HIV patients [Meyer-König et al., 1998].

The results indicate that previous infections with a CMV gB genotype does not protect renal transplant recipients from a reinfection with another gB genotype. These data are of great interest for selecting a candidate vaccine. Probably, vaccines of recombinant gB or attenuated viruses having only one gB genotype will not protect renal transplant patients against all the CMV genotypes.

An association of gB genotypes was not found with symptomatic CMV infection in the renal transplant recipients, analyzing either individual gB genotypes or the combination of them. Despite the relatively small number of patients included in this study, these data are in line with previous studies of renal transplant recipients, that showed no correlation between gB genotypes and the development of symptomatic CMV infection [Vogelberg et al., 1996; Woo et al., 1997]. On the other hand, studies with bone marrow transplant patients have shown a correlation of gB 2 with CMV disease and gB 3 and gB 4 with death due to myelosuppression [Fries et al., 1994; Torok-Storb et al., 1997; Woo et al., 1997]. In AIDS patients, Shepp et al. [1996] observed an association of gB2 and retinitis that was not confirmed by Peek et al. [1998]. Bongarts et al. [1996], however, found the gB 1 genotype less frequently in retinitis patients as compared to HIV patients with asymptomatic CMV infection. Probably, the immunological state of renal transplant recipients is capable of controlling CMV infection as compared to the bone marrow transplant and AIDS patients, thus the genotype differences would be not important in these types of patients.

In conclusion, this study showed that the renal transplant recipients are usually infected with more than 1 CMV gB genotype and that the gB genotypes did not correlate with the development of symptomatic CMV infection.

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